Somatostatin analog Lanreotide inhibits myocyte replication and several growth factors in allograft arteriosclerosis

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ABSTRACT **Chronic rejection is the most common reason for late loss of a transplant. The molecular mechanism of chronic rejection is not known and there is no treatment for this disorder. The characteristic histological feature in chronic rejection is increased smooth muscle cell replication in the vascular wall, leading to allograft arteriosclerosis. In this study we demonstrate that nonimmunosuppressed rat aortic allografts undergoing chronic rejection synthesize increased quantitites of several smooth muscle cell growth-promoting substances in the vascular wall including interleukin-1, eicosanoids, and several peptide growth factors. Administration of a stable somatostatin analog lanreotide, BIM 23014, strongly inhibits myocyte proliferation in the allograft in vivo. It has no inhibitory effect on the proliferation of smooth muscle cells in vitro. Concomitantly, the locally produced peptide growth factors, i.e., epidermal growth factor, insulin-like growth factor 1, and BB-isomer of platelet-derived growth factor, but not other mediators of inflammation, are significantly reduced. The results suggest that growth factors are the main effector molecules leading to myocyte proliferation in allograft arteriosclerosis and that allograft arteriosclerosis (chronic rejection) may be specifically inhibited by lanreotide administration.—Häyry, P., Räisänen, A., Ustinov, J., Mennander, A., Paavonen, T. Somatostatin analog lanreotide inhibits myocyte replication and several growth factors in allograft arteriosclerosis. *FASEB J.* 7: 1055-1060; 1993.**

Key Words: allograft arteriosclerosis · chronic rejection · smooth muscle cell · growth factors

THE DISCOVERY OF AN INHIBITOR OF GROWTH hormone release in sheep hypothalamus (1), later called somatostatin, has led to the development of more stable analogs for clinical use. Certain octapeptide analogs of somatostatin have been reported effective in the treatment of acromegaly (2), in the inhibition of gastrointestinal secretion (3), tumor growth (4, 5), and in diabetic angiopathy (6).

The beneficial effect of somatostatin analogs on diabetic angiopathy is probably due to their inhibitory effect on angiogenesis. The same mechanism may be at least partially responsible for the prevention of tumor growth (7). Subsequently, it has also been demonstrated that analogs of somatostatin, including lanreotide, inhibit myointimal proliferation after air drying injury in the rat (8) and balloon injury in the rabbit (9). Upon comparing the relative efficacy of several different analogs of somatostatin in the air drying injury model, lanreotide BIM 23014 (angiopeptin) proved particularly effective (8). BIM 23014 has also been shown to inhibit chronic coronary vascular disease in rabbit (10) and rat (11) cardiac transplant models.

The common feature in all allografts undergoing chronic rejection is persistent perivascular inflammation and increased smooth muscle cell replication in the vascular wall, leading to concentric longitudinal intimal thickening, called allograft arteriosclerosis (12, 13). In this communication we demonstrate that vascular smooth muscle cell replication in allograft arteriosclerosis is accompanied by the synthesis of several peptide growth factors in the vascular wall. Administration of somatostatin analog lanreotide, BIM 23014, selectively reduces the growth factor synthesis in the vascular wall and, concomitantly, blocks the vascular smooth muscle cell replication.

MATERIALS AND METHODS

Smooth muscle cells (SMC)[2] were isolated from aortas of 9- to 11-day-old DA rats according to Thyberg et al. (14). The aortas were opened longitudinally, the intimal layer was gently scraped off, and the media were carefully separated and sliced into small pieces. The media were digested with 0.1% collagenase and 0.02% DNAse in PBS at +37°C for 1-2 h. Isolated cells from the media were centrifuged, suspended in culture medium (Dulbecco's modified Eagle's medium supplemented with 10% FCS; Sera-Lab, Sussex, U.K.) with 2 μmol/ml glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin, seeded in plastic flasks, and incubated at +37°C with 5% CO_2 in air. After one or two passages in bulk cultures in vitro, the SMC were seeded in 96-well multidishes (8000 cells/well) in full culture medium supplemented with BIM 23014 ("angiopeptin," Henri Beafour Inst. USA Inc., Washington, D.C.) at different concentrations (0-1000 nmol/l). The cells were exposed daily to 1 μCi/ml of [^{3}H]thymidine or [^{3}H]glycine for 24 h to quantitate DNA and protein synthesis, respectively. After each 24 h pulse, the cells were washed three times with PBS, detached with 1.25% trypsin, and mixed with OptiPhase "HiSafe" 3 (LKB-Wallac, Turku, Finland). The radioactivity was measured with a Rackbeta liquid scintillation counter (LKB-Wallac).

WF (AGB2-RT1^v) and DA (AGB4, RT1^a) rats (Zentralinstitut fur Versuchstierzuht GmBH, Hannover, Germany) weighing 200-300 g were anesthesized with chloralhydrate (6 ml/kg) i.p. A segment of the descending thoracic aorta, approximately 3 cm in length, was excised, perfused, and used

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[2]Abbreviations: SMC, smooth muscle cells; RIA, radioimmunoassay; EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; PDGF-BB, plate-derived growth factor B-chain.

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as a transplant (15). The grafts were transplanted end-to-end using 9-0 continuing nylon suture below renal arteries and above bifurcation, forming a "loop" in the abdominal cavity of the recipient. The grafts were removed at 10 and 20 days, and 1, 2, 3, 5, and 6 months after transplantation and processed for histology, autoradiography, and immunochemical determinations (see later).

BIM 23014C was dissolved in water and administered with Alzet osmotic minipumps (Model 2002; Alza Corp., Palo Alto, Calif.) at a flow rate of 80 $\mu g \cdot kg^{-1} \cdot day^{-1}$. At each replacement, the pumps were examined to ensure that they had functioned flawlessly.

The rats received 250 μCi of tritium-labeled thymidine ([^{3}H]TdR; NEN Chemicals, Boston, Mass.) by i.v. injection 3 h before death. Emulsion autoradiography (Ilford L.4; Ilford, Mobberley, Cheshire, England) was processed from paraffin sections. The results were quantitated as number of labeled nuclei/layer using cross-sectional areas of the aorta. For immunohistochemistry-autoradiography the specimens were immersed in Tissue-Tek (Miles Inc., Elkhard, Ind.) and snap-frozen.

For immunochemical determinations, aortic allografts and syngeneic grafts as well as thoracic and abdominal aortas of the recipient or a normal control rat were removed and sliced. Aortas were first incubated for 30 min in an ice bath with 0.1 M Na-phosphate buffer, pH 7.4, in 0.9% NaCl supplemented with 1% BSA, 200 nM Ca-ionophore (Sigma, St. Louis, Mo.) and 0.1% NaN_3, and thereafter for another 30 min at +37°C in the same buffer (1-3 ml). Exclusion of NaN_3 and/or Ca-ionophore from the incubation medium (which was present in the preliminary experiments) did not affect the determinations. Incubation was discontinued by taking the aortas back to the ice bath. Liquid from the incubation tubes was collected for radioimmunoassay (RIA) and the aortas were dried overnight at +37°C and weighed. Commercially available RIA kits (Amersham, Buckinghamshire, U.K.) for interleukin-1 (IL-1), 6-keto-PGF1α for prostacyclin, and TxB2 for thromboxane A2, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and platelet-derived growth factor B-chain (PDGF-BB) were used. The data are given as nanogram or picogram per milligram aorta dry weight.

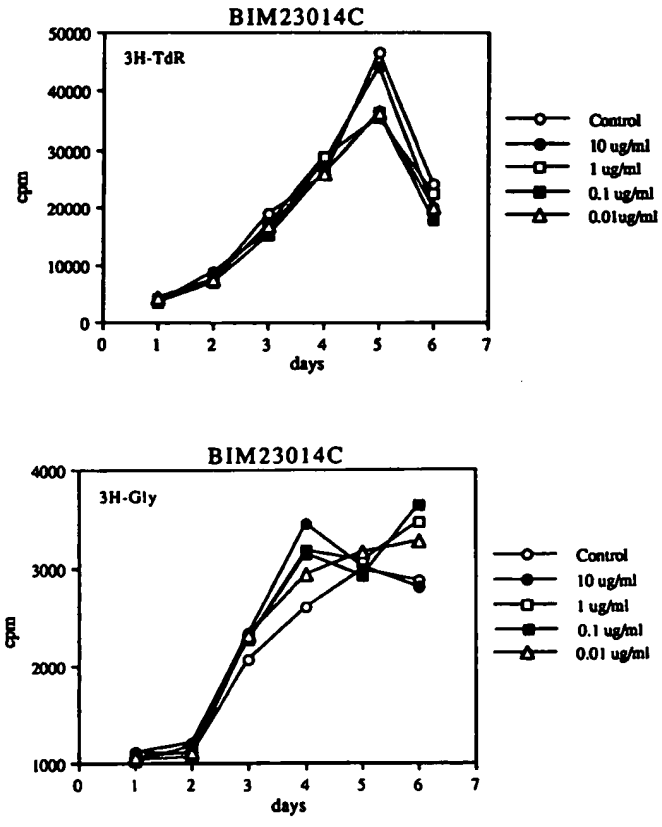

Figure 1. The effect of the somatostatin analog lanreotide, BIM23014, on the replication ([^{3}H]TdR) and protein synthesis ([^{3}H]glycine) of freshly isolated smooth muscle cells in vitro.

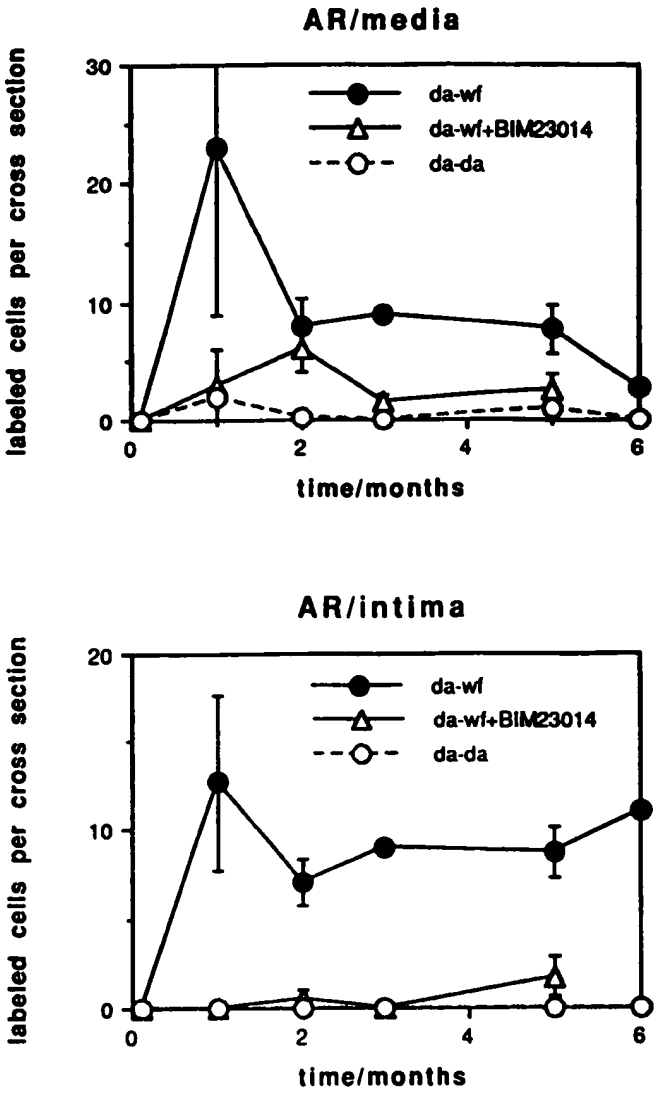

Figure 2. Effect of somatostatin analog lanreotide, BIM 23014, on the proliferative response of smooth muscle cells (SMC) in the media and intima of DA to WF allografts. For comparison, the proliferation of smooth muscle cells in DA to DA syngeneic grafts is also shown.

RESULTS

As somatostatin analogs were previously shown to be inhibitory both to intimal thickening after air drying carotid injury and cardiac transplantation in the rat, which both represent smooth muscle cell proliferative responses in vivo, we first

tested the potency of the most effective somatostatin analog lantreotide, BIM 23014, on smooth muscle cell replication in vitro. BIM 23014 was applied to smooth muscle cell cultures over a very wide concentration range, and the responses were compared with control cultures not containing the drug by tritiated thymidine ([^{3}H]thymidine) incorporation (for DNA synthesis) and by [^{3}H]glycine incorporation (for protein synthesis).

As seen in **Fig. 1**, BIM 23014 did not inhibit smooth muscle cell replication in vitro. None of the tested concentrations

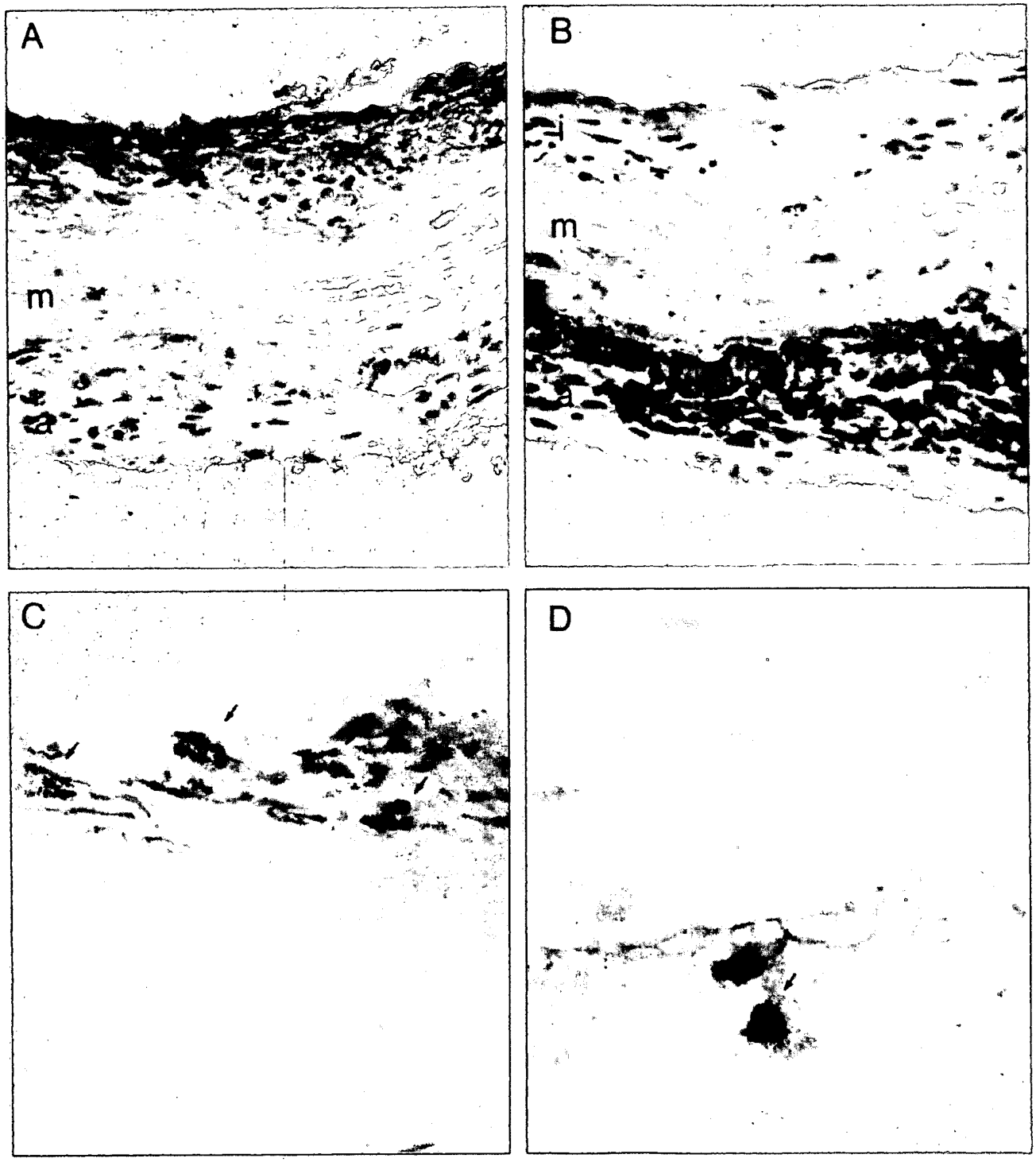

Figure 3. Upper section: a microphotograph of aorta allograft vascular wall demonstrating peroxidase staining of anti-smooth muscle actin in the intima (*A*) and anti-leukocyte common antigen in the adventitia (*B*) in two separate sections of the same area. Three three layers of the aorta are indicated as: i, intima; m, media; a, adventitia. Approx. 100×. Lower section: Identification of [^{3}H]TdR incorporating cells with anti-alpha actin antibody in the intima (*C*) and with anti-leukocyte common antigen antibody in the adventitia. (*D*). Arrows indicate the double-labeled cells. Approx. 300×.

of the drug were toxic to the cells. The results were similar if vascular adventitial fibroblasts instead of smooth muscle cells were used (not shown). The results were also similar if a somatostatin analog octreotide, SMS201995 ("sandostatin"), was used instead of the lanreotide (not shown).

In the second experiment rat aortic allografts were transplanted from DA to WF strain and left nonimmunosuppressed. We have previously demonstrated (15) that aorta transplants between histoincompatible rat strains may be used as an animal model for allograft arteriosclerosis: the histopathological alterations in aortic allografts closely resemble those observed in human transplant arteries during chronic rejection, and these changes are not recorded in syngeneic control transplants.

To quantitate the proliferative response of smooth muscle cells in the vascular wall, the recipient animals received 250 μCi of [^{3}H]TdR 30 min before death, and autoradiograms were performed from paraffin sections.

As seen in **Fig. 2**, at 1-5 months after transplantation there was a strong proliferative response in the allograft media and intima. In syngeneic controls, practically no proliferation was observed. Both in the media and intima, the proliferation peaked shortly after the transplantation. In the media it declined thereafter, whereas in the intima the proliferation continued until the end of the experiment.

Autoradiograms were also performed from frozen sections of the vascular wall, and stained with monoclonal antibodies to alpha smooth muscle cell actin or to leukocyte common antigen prior to the procedure (**Fig. 3**). Double labeling demonstrated that of the [^{3}H]TdR incorporating cells in the intima, 81% were alpha smooth muscle cell actin antibody-reactive, 13% reacted with the antibody to common leukocyte antigen, and 6% with neither. Most of the double negative cells in the intima were identified, due to their localization and morphology, as endothelial cells.

When the recipient animals received 80 $\mu g \cdot kg^{-1} \cdot day$ of BIM 23014 with osmotic minipumps, the proliferative response of smooth muscle cells in the media was reduced by more than 50%, and in the intima close to the zero level of syngeneic controls (Fig. 2).

We also quantitated the effect of BIM 23014 administration on the histology of the allograft intima from hematoxylin-eosin stained paraffin sections. BIM 23014 administration significantly ($P < 0.05$) delayed the generation of intimal thickness in the nonimmunosuppressed recipients by nearly 50% (**Table 1**).

In the final experiment we quantitated several cytokines, eicosanoids, and growth factors, previously reported to be growth-promoting to vascular smooth muscle cells in vitro (14), from the vascular wall and the effect of BIM 23014 administration on them. The transplantations were performed as above, the middle section of the transplant was cut in small slices, incubated in tissue culture medium in vitro, and the release of various mediators of inflammation was quantitated from the medium by conventional RIA assays for interleukin-1 (IL-1), 6-keto-PGF1α, thromboxane-B2 (TxB2), leukotriene B4 (LTB4), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor BB-isomer (PDGF-BB), and endothelin (type 3).

TABLE 1. *Intima thickness in morphological point score units (psu)[a] in DA to WF allografts, where the recipient rats received 80 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of BIM23014 as continuous infusion or were left nontreated[b]*

	Intima response in psu (time posttransplantation)		
	1 month	2 months	6 months
DA → WF	1.5 ± 0.3 (n = 21)	2.9 ± 0.1 (n = 22)	5.5 ± 0.6 (n = 7)
DA→WF + BIM23014	0.9 ± 0.4 (n = 7)	0.7 ± 0.3 (n = 3)	3.1 ± 0.6 (n = 4)
	ns	$P < 0.05$	$P < 0.05$

[a]For quantitation of histology in psu, see ref 15.
[b]Significances according to Mann-Whitney test, *ns*, nonsignificant.

As seen in **Table 2**, allografts contained increased quantities of IL-1 compared to syngeneic controls. There was a nearly 2.5-fold increase in the level of TxB2 in the allografts compared to syngeneic controls, a small and insignificant decrease in the level of 6-keto-PGF1α, and no increase in LTB4. A very significant increase in the level of EGF, IGF-1, and PDGF-BB was observed in the allografts compared to syngeneic controls whereas no increase in the level of rat type endothelin-3 was seen.

The location of the molecules, whether present mainly in the inflammatory adventitia or in the intima, was investigated further by microdissecting these two vascular layers apart and by separate determination of the molecules from tissue extracts of these two vascular wall components. As also shown in Table 2, thromboxane was present predominantly in the vascular adventitia (and presumably synthesized by the inflammatory cells; adventitia/intima ration >1). Instead, 6-keto-PGF1α and the growth factors EGF, IGF-1, and PDGF-BB were recovered mainly in the intima (presumably synthesized by the endothelial and smooth muscle cells and inflammatory macrophages; ratio <1). IL-1 was present both in the adventitia and intima (ratio 1.2).

Administration of BIM 23014 significantly reduced the levels of EGF ($P < 0.005$), IGF-1 ($P < 0.05$), and PDGF-BB ($P < 0.01$) in the vascular wall (**Table 3**). It failed to reduce the levels of IL-1, TxB2, or 6-keto-PGF1α (P ' ns).

TABLE 2. *The synthesis of certain cytokines, eicosanoids and growth factors in the whole wall of a normal aorta, syngenenic graft, and allograft, and the ratio of synthesis of these mediators in the allograft adventitia vs. intima[a]*

	Units	Normal aorta	DA-DA syng. graft	DA-WF allograft	Adv./intima ratio in DA-WF allograft
IL-1	pg/mg	0.6 ± 0.2	0.8 ± 0.1	1.6 ± 1.2	1.2
6-keto-PGF1a	ng/mg	16.3 ± 1.7	16.8 ± 2.3	13.9 ± 3.5	0.5
TxB2	ng/mg	0.2 ± 0.2	2.3 ± 0.4	5.5 ± 6.8	5.7
LTB4	ng/mg	<2[b]	<2[b]	2[b]	nd[c]
EGF	pg/mg	80 ± 15	165 ± 20	593 ± 30	0.8
IGF-1	pg/mg	460 ± 90	1492 ± 161	4811 ± 120	0.7
PDGF-BB	pg/mg	≤50	≤50	460 ± 49	0.3
Endothelin	pg/mg	14.0 ± 3.1	9.1 ± 5.9	11.6 ± 2.7	nd

[a]During early chronic stage, 2-3 months posttransplantation, ± SEM. [b]Detection limit of the assay. [c]nd, not done.

TABLE 3. *Inhibition of cytokine, eicosanoid, and growth factor synthesis by BIM 23014 in the allograft*

	Units	DA-WF allograft (n[a])	DA-WF allograft + BIM 23014 (n)	% of nontreated allograft	P[b]
IL-1	pg/mg dry wt	(6) 1.6 ± 1.2	(5) 1.9 ± 0.2	118%	ns
TxB2	ng/mg dry wt	(7) 5.5 ± 6.8	(8) 5.4 ± 1.2	100%	ns
6-keto-PGF1a	ng/mg dry wt	(7) 13.9 ± 3.5	(7) 16.5 ± 1.9	123%	ns
EGF	pg/mg dry wt	(5) 593 ± 30	(5) 376 ± 42	68%	<0.005
IGF-1	pg/mg dry wt	(5) 4811 ± 120	(5) 3625 ± 485	75%	<0.05
PDGF-BB	pg/mg dry wt	(5) 460 ± 49	(5) 160 ± 35	35%	<0.01

[a] n indicates number of rats killed for each determination. [b] Significances by impaired Student's t test. P values >0.05 are considered nonsignificant (ns).

DISCUSSION

The physiological effects of somatostatin analogs have previously been accredited either to the inhibition of growth hormone secretion from the hypothalamus or to the inhibition of growth factor secretion, particularly of IGF-1 (16, 17). Alternative mechanisms have also been suggested, e.g., a direct inhibitory effect on the mitogenesis and/or an inhibitory effect via a third molecule, such as IGF binding protein-1, IGFbp-1 (17).

In this communication we demonstrate for the first time a pronounced inhibitory effect of somatostatin lanreotide analog on the replication of smooth muscle cells in the vascular wall during chronic rejection. Our model also made it possible to investigate the molecular mechanism of allograft arteriosclerosis and to reinvestigate the site (or sites) of lanreotide inhibitory effect.

Previous in vitro studies, summarized by Thyberg et al. (14), have linked at least three separate sets of molecules with the regulation of smooth muscle cell replication in vitro: interleukins-1, and -6, eicosanoids and polypeptide growth factors, particularly EGF, IGF-1 and PDGF-BB, and endothelin. Considering the molecular mechanisms of lanreotide inhibition, the following possibilities exist: direct inhibition of smooth muscle cell replication and/or the inhibition of one or more of these growth regulatory molecules.

Our in vitro data show that lanreotide had no effect on the replication of smooth muscle cells in vitro. Thus, a direct inhibition of smooth muscle cell mitogenesis may be a less likely explanation.

Our determinations of the mediators of inflammation demonstrated that IL-1 and TxB2 were significantly elevated during allograft arteriosclerosis whereas there was no change in the level of 6-keto-PGF1α or LTB. Administration of lanreotide brought down none of them. The levels of all measured growth factors for which RIA-assays are available in the rat, i.e., EGF, IGF1, and PDGF-BB, were also significantly elevated during allograft arteriosclerosis. They all were also down-regulated by lanreotide administration. Thus our findings clearly suggest that smooth muscle cell replication in allograft arteriosclerosis is controlled via several peptide growth factors present mainly in the allograft intima, and vice versa, it appears that the inhibitory effect of lanreotide analogs of somatostatin is based on the inhibition of these growth factors.

More recently somatostatin and some of its analogs, including lanreotide BIM 23014, have been shown to activate a membrane-bound (phosphoryl protein tyrosine) phosphatase (18, 19) that dephosphorylates the activated tyrosine kinase domains of the receptors for a series of growth factors like EGF, IGF-1, and PDGF (20). It was not possible to judge this additional site of lanreotide effect in the present study, but it is worth examining later.

Taken together, our results demonstrate that a lanreotide analog of somatostatin significantly suppresses smooth muscle cell replication in allograft arteriosclerosis (chronic rejection) and delays the generation of arteriosclerotic changes in nonimmunosuppressed recipients. As the levels of several growth factors were concomitantly reduced in the vascular wall, the results indicate that growth factors are the final effector molecules of smooth muscle cell replication in allograft arteriosclerosis. These growth factors are most likely locally produced, as no such alterations in the vascular wall, nor any synthesis of these growth factors, were ever observed in other parts of the recipient vasculature except in the allograft itself. It is therefore likely that their production is under the control of the adventitial immune inflammation. If lanreotide administration will also reduce the "secondary" changes (12) in other experimental transplants, such as glomerular sclerosis and tubular atrophy in kidney transplants, they may become the first experimental molecule to counteract chronic rejection in organ transplantation in humans. FJ

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